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In vitro kinase assay

Vladimir Denic HY Houqing YU

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An abbreviated version of this protocol was published in eLIFE in Apr 2022

The peroxisomal exportomer directly inhibits phosphoactivation of the pexophagy receptor Atg36 to suppress pexophagy in yeast

DOI: 10.7554/eLife.74531

Detailed protocol

In vitro kinase assay

Used in Yu *et al*., *Elife*. 2022 Apr 11;11:e74531.

1. Prepare 20× kinase buffer, aliquot and store at -20 ^{o}C.
   20× kinase buffer: 1 M Tris-HCl pH 7.5, 2 mM EDTA and 40 mM DTT
2. Prepare fresh 10× ATP regeneration system (ARS) for each assay, keep on ice.
   10× ARS: 100 mM $MgCl_2$, 10 mM ATP, 100 mM creatine phosphate and 2 mg/mL creatine kinase
3*. Prepare 4× kinase mixture, keep on ice
   0.8 μM Hrr25 (wild type or K38A mutant), 0.8 μCi/μL γ-^{32}P-ATP, 4× ARS and 4× kinase buffer;
   used at 3 μL per 12 μL reaction in PCR tubes
4. Prepare 4× substrate mixture, keep on ice
   2 μM Atg36 or 4 μM Atg19, or 2 μM Atg36 and Atg19 diluted with 25 mM HEPES pH 7.5, 150 mM NaCl, and 1 mM DTT
   used at 3 μL per 12 μL reaction in PCR tubes
5. Prepare 2× ATPase mixture, keep on ice
   400 nM ATPases (wild-type Pex1/6 and Walker B mutant (E832A), wild-type Cdc48 and D2 mutant (E588Q)
   used at 6 μL per 12 μL reaction in PCR tubes.
6. Mix 4× substrate with 2× ATPase mixture, pre-incubate the substrate/ATPase mixture on a 37 ^{o}C heating block for 5 min
7*. Pre-incubate the 4× kinase mixture on a 37 ^{o}C heating block for 5 min
8*. Add substrate/ATPase into kinase mixture to start the reaction
9*. Take 2 μL sample at designed time points (e.g., 0.5, 3, 6, 10 and 15 min), mix the samples immediately with 15 μL 1× SDS-PAGE loading buffer, 95 ^{o}C denaturation for 5 min
10*. Load the sample (~17 μL) onto SDS-PAGE gels (Novex™ WedgeWell™ 4 to 20%, Tris-Glycine, 1.0 mm, Mini Protein Gels), electrophoresis at 200 V for 55 min
11*. Soak the protein gels in gel fixing solution (50% v/v ethanol and 10% v/v acetic acid in water), shake at room temperature for 15 min.
12*. Soak the protein gels in gel drying solution (45% v/v ethanol and 5% v/v glycerol in water), shake at room temperature for 15 min.
13*. Dry the gels with Model 583 Gel Dryers (Bio-Rad)
14*. Expose the dried gels to phosphorimager cassettes overnight
15. Quantify the ^{32}P-labeled species of Atg36 or Atg19 with FIJI software.

*. ^{32}P related operation. wear appropriate personal protective equipment

How to cite:(Readers should cite both the Bio-protocol preprint and the original research article where this protocol was used)

1. Denic, V. and YU, H. (2022). In vitro kinase assay. Bio-protocol Preprint. bio-protocol.org/prep1895.
2. Yu, H., Kamber, R. A. and Denic, V.(2022). The peroxisomal exportomer directly inhibits phosphoactivation of the pexophagy receptor Atg36 to suppress pexophagy in yeast. eLIFE. DOI: 10.7554/eLife.74531